Immunology 1991 **73** 472–477 ADONIS 001928059100184L

IL-8 causes *in vivo* neutrophil migration by a cell-dependent mechanism

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Accepted for publication 23 April 1991

SUMMARY

A dose-dependent neutrophil migration was observed following the injection of recombinant interleukin-8 (rIL-8) into rat peritoneal cavities. This finding contrasts with the inability of rIL-8 to induce neutrophil emigration into subcutaneous air-pouches. Pre-treatment of the animals with dexamethasone (0·5 mg/kg, s.c.) or depletion of the peritoneal resident cell population abolished the neutrophil migration induced by rIL-8 and by recombinant interleukin-1β (rIL-1β). Different from that which occurs with rIL-1β, neutrophil migration induced by rIL-8 was not enhanced by an increase in the peritoneal macrophage population. Transference of homologous total resident peritoneal cells to the air-pouch rendered this cavity responsive to the chemotactic effect of rIL-8 and potentiated the neutrophil migration induced by rIL-1. Our results show that both rIL-8 and rIL-1β are able to induce *in vivo* neutrophil migration by an indirect mechanism, dependent on resident cells. Neither macrophages nor lymphocytes seem to be involved in the rIL-8 chemotactic effect. However, peritoneal resident mast cells may be implicated in this mechanism. These cells, when stimulated *in vitro* by rIL-8, released a factor that when injected into peritoneal and air-pouch cavities induced neutrophil migration.

INTRODUCTION

Different laboratories, including our own, have shown that *in vivo* neutrophil migration induced by human interleukin-1 (IL-1),[1,2] tumour necrosis factor (TNF-α)[2,3] and interferon-gamma (IFN-γ)[4] does not result from a direct chemoattractant activity but is mediated via the release of chemotactic factors from resident macrophages.

Recently, a neutrophil-activating protein has been purified[5,6] and cloned[7] and named interleukin-8 (IL-8). IL-8 is released by various cell types, including those of the phagocyte mononuclear system,[8,9] endothelial cells[10] and T lymphocytes.[11] Although rIL-8 has anti-inflammatory activity through inhibition of neutrophil adhesion to activated endothelial cells,[10] studies from several laboratories implicated this cytokine as a pro-inflammatory mediator. IL-8 has been found in inflammatory exudates[12] and promotes several inflammatory events such as hyperalgesia,[13] histamine release,[14] neutrophil degranulation,[15] respiratory burst activation,[16] neutrophil chemotaxis[5,6] and *in vivo* neutrophil emigration into the skin of rabbit.[16,17]

The purpose of the present study was to investigate whether human recombinant IL-8 causes neutrophil migration into the peritoneal cavity and into the skin air-pouch of rats and whether this effect, as has been described for rIL-1, is dependent on resident cells. For this purpose, we tested this cytokine's ability to induce neutrophil migration into normal rat abdominal cavity and into cavities where the resident cells were reduced by lavage or the macrophage population was increased by thioglycollate pretreatment. Because it has been shown that in these tests corticoids are able to inhibit neutrophil migration by blockade of the release of chemotactic substances by resident cells stimulated by various stimuli,[18] we also studied the action of dexamethasone upon rIL-8-induced neutrophil migration. The reduction of rIL-8-induced neutrophil migration by peritoneal lavage and corticoid pretreatment suggested the participation of resident cells. rIL-8 was also tested in the rat skin air-pouch models. In these tests rIL-8 failed to produce neutrophil migration. However, after transference of peritoneal homologous cells to 6-day-old pouch, this cavity became responsive to rIL-8. This result further supports the suggestion that rIL-8-induced neutrophil migration *in vivo* is indirect and is mediated by resident cells. Among the resident cells it was found that mast cells released a chemotactic factor which caused *in vivo* neutrophil migration.

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MATERIALS AND METHODS

Animals

Male Wistar rats weighing 180–200 g were housed in temperature-controlled rooms and received water and food *ad libitum* until use.

Chemotactic stimuli

Human recombinants IL-8 (Lot number 89/520) and IL-1β [Lot number 86/526A; National Institute for Biological Stan-

dards and Control (NIBSB) Herts, U.K.] and FMLP (Sigma, St Louis, MO) were used as neutrophil chemotactic stimuli.

In vivo *neutrophil migration induced by cytokines*

Peritoneal cavities. rIL-8 (30 and 60 ng/cavity) or rIL-1β (75 pg/cavity) was diluted in 3 ml of sterile phosphate-buffered saline (PBS) and injected intraperitoneally (i.p.) in normal rats. The highest dose of rIL-8 (60 ng) and 75 pg of rIL-1β were also injected into rats pretreated 1 hr earlier with dexamethasone (Merck Sharp & Dohme, São Paulo, Brazil) (0·5 mg/kg, s.c.). Control animals received 3 ml of PBS i.p.

Air-pouches. Rat skin 6-day-old air-pouches were produced as described previously.[19] The backs of the rats were shaved and 20 ml of sterile air were injected subcutaneously. Three days later, 10 ml of sterile air were again injected to maintain pouch patency. Six days after the initial injection the pouches were used. rIL-8 (6, 60 and 120 ng/pouch) or rIL-1β (75 pg/pouch) was diluted in 1 ml of sterile PBS and injected into 6-day-old air-pouches of normal rats. rIL-8 (120 ng/pouch) or rIL-1β (75 pg/pouch) was also injected into 6-day-old air-pouches of rats pretreated with dexamethasone 1 hr earlier (0·5 mg/kg, s.c.). Control animals received 1 ml of PBS.

Collection of exudates

Four hours after injection of the test solutions into peritoneal cavities or 6 hr after injection into air-pouches, the animals were killed and the peritoneal cavities or air-pouches were harvested by injection of 10 or 5 ml of PBS containing 5 U/ml of heparin, respectively. Total and differential cell counts were performed as described elsewhere.[20] The results were reported as the number of cells per cavity.

Changes in peritoneal macrophages population

Increase of peritoneal macrophages by thioglycollate pretreatment. Thioglycollate (Lab. Difco Ltda, São Paulo, Brazil) (Tg, 3% w/v, 10 ml) was injected i.p. in a group of rats and after 4 days peritoneal macrophages were collected from half of those rats, counted, and compared with the number of the same cells obtained from a group of non-treated rats (control). rIL-8 (30 ng/3 ml) and rIL-1β (75 pg/3 ml) were then injected into the remaining rats (control and Tg-treated), and after 4 hr neutrophil migration induced by both recombinant cytokines was evaluated as described earlier. At the time of the macrophage count (4 days after Tg), a residual number of neutrophils ($7{\cdot}6 \pm 0{\cdot}22 \times 10^6$/cavity; $n=5$) was detected in the peritoneal washes. To avoid a possible bias, this mean value was subtracted from the number of neutrophils appearing after rIL-8 and rIL-1β administration in Tg-treated animals.

Depletion of peritoneal macrophages by peritoneal lavage. The method employed has already been described.[21] Briefly, male Wistar rats were anaesthetized with ethyl ether and three hypodermic needles were inserted into the abdominal cavity. Sterile saline (30 ml) was injected through the needle placed near the sternum. The abdominal cavity was then gently massaged for 1 min and the peritoneal fluid collected via the two needles inserted into the inguinal region. This operation was repeated three times. More than 85% of the peritoneal macrophage population was recovered in the lavage fluid and 95% of the injected saline was recovered. If blood was detected visually in the lavage fluid the animal was discarded. Control (Sham) rats were impaled and manipulated in the same way but no fluid was injected or withdrawn. Thirty minutes later, the peritoneal macrophage population was estimated in half of those rats by injecting 10 ml of PBS–heparin–BSA, as described above. The other half of each group received rIL-8 (30 ng/3 ml), rIL-1β (0·15 ng/3 ml) and FMLP (4·5 ng/3 ml) and neutrophil migration was estimated 4 hr later.

Homologous transference of total peritoneal resident cells to 6-day-old air-pouch

Total peritoneal resident cells (TPC) were obtained by peritoneal lavage (see above) with sterile heparinized-RPMI (5 UI/ml). The peritoneal washes were centrifuged (300 ***g***, 10 min at 4°) and the cells washed twice and resuspended to obtain 10^6 cells/ml of RPMI. The animals received 1 ml of the cell suspension/pouch (TPC group) or 1 ml of RPMI (Sham-washed; S group). After 15 min, RPMI (1 ml), rIL-8 (75 ng/1 ml of RPMI) or rIL-1β (75 pg/1 ml of RPMI) was injected into the air-pouches. Six hours after stimuli injection neutrophil migration was evaluated.

Release of neutrophil chemotactic factor in vitro *from mast cells, lymphocytes or macrophages stimulated with rIL-8*

Mast cell purification

The method used was based on the centrifugation of peritoneal cells by Percol density gradient separation.[22] Briefly, all peritoneal cells of 25–35 male rats weighing 250–300 g were collected by lavage of the peritoneal cavity of each rat with 10 ml Krebs' Ringer Phosphate Buffer (KRP). The cells, originating from three animals, were pooled and centrifuged in a polypropylene tube at 300 ***g*** for 5 min at 4°. The supernatant was discarded and the cell pellets were resuspended in 0·75 ml KRP, and 3·5 ml Percoll (300 mOsm/kg H_2O) was added and gently mixed. The cells were allowed to settle for 10 min and centrifuged at 750 ***g*** for 15 min after the addition of 0·5 ml of KRP. The upper KRP phase as well as the cells present in the Percoll medium interface were carefully removed by aspiration and discarded. The lower Percoll phase was diluted with 10 ml of KRP and the tubes were further centrifuged at 300 ***g*** for 5 min at 4°. The cell pellets were washed twice with KRP and resuspended in 1 ml of KRP. The purity of the mast cell suspension was determined by counting the cells by conventional light microscopy after staining with toluidine blue.

Purified mast cells (10^6/tube) were incubated with shaking with 4 ml of KRP (control) or IL-8 (20 ng/ml) during 30 min at 37°. After incubation, the tubes were centrifuged at 300 ***g*** for 5 min at 4°. The pelleted cells were washed twice with KRP and incubated for 1 hr with PBS without the cytokines. The supernatants were ultrafiltrated in Amicon YM-5 membrane (Grace Co., Beverly, U.S.A.) and the retentate fraction was resuspended in PBS. The dilutions were made in order to obtain a solution in which 1 ml was originated from $1{\cdot}25 \times 10^5$ mast cells. For testing the possible release of a chemotactic factor, 1 or 3 ml of the solution were injected into air-pouch or peritoneal cavities, respectively, of the naive rats. The neutrophil emigration was analysed 4 (peritoneal cavity) or 6 (air-pouch) hr after, as described above.

Lymphocyte isolation

Lymphocytes were collected over 2–3 hr from anaesthetized naive rats, through a cannula implanted in the thoracic duct.[23] The cells were separated from lymph by centrifugation and were

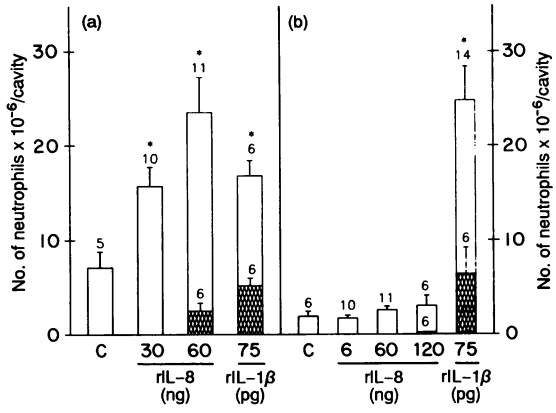

Figure 1. rIL-8 induces neutrophil migration into peritoneal cavities but not into 6-day-old air-pouches. The bars represent neutrophil migration into peritoneal cavities (a) or into air pouches (b) induced by vehicle (C), rIL-8 and rIL-1β in PBS (open bars) or dexamethasone (0·5 mg/kg, s.c. 1 hr before; crosshatched bars) pretreated rats. The results are reported as mean ± SEM. The number of animals utilized is indicated at the top of each column. $*P<0.05$ compared to control animals (C); Student's t-test.

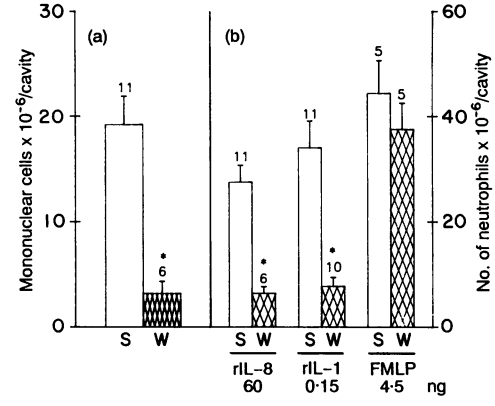

Figure 2. The reduction of the resident cell population of rat peritoneal cavities inhibits neutrophil migration induced by rIL-8 and rIL-1β, but not by FMLP. (a) Shows the number of mononuclear cells in sham (S; open bar) and washed (W; crosshatched bar) rat peritoneal cavities. (b) Shows neutrophil migration induced by 60 ng of rIL-8, 0·15 ng of rIL-1β and 4·5 ng of FMLP into sham (S) and washed (W) cavities. The results are reported as mean ± SEM. The number of animals utilized is indicated at the top of each column. $*P<0.05$ compared to sham-washed animals (S); Student's t-test.

washed three times in PBS. The lymphocytes (2×10^7/tube) were incubated during 2 hr at 37° with 4 ml of RPMI alone (control) or containing rIL-8 (20 ng/ml). After this period of time, the supernatants were discarded, the cells were washed twice and further incubated with the medium for 2 hr. The supernatants were ultrafiltered in YM-5 membrane (cut-off 5000 MW) and the retentate resuspended in PBS. The dilutions were made in order to obtain a solution in which 1 ml was originated from 2.5×10^6 lymphocytes and the assays were made as described above for mast cells.

Macrophages isolation

Rat peritoneal macrophages were harvested with RPMI (pH 7·4) 4 days after i.p. injection of thioglycollate (3% w/v, 10 ml) and cultured in plastic tissue culture dishes as previously described.[18] After 1 hr of incubation, non-adherent cells were removed by washing three times with RPMI medium. The adherent population, consisting of 95% macrophages, was incubated for 30 min at 37° in fresh medium (control) or medium containing rIL-8 (20 ng/ml). After that, the supernatants were discarded and after further washings the cells were incubated for 1 hr with 4 ml of medium, without the cytokine. The cell-free incubation fluids were ultrafiltered in YM-5 membrane and the retentate resuspended in PBS. The dilutions were made in order to obtain a solution in which 1 ml was originated from 3×10^6 macrophages and the assays were made as described above.

RESULTS

Comparison of (a) and (b) of Fig. 1 shows that intraperitoneal injection of rIL-8 caused a dose-dependent neutrophil emigration into peritoneal cavities, while its administration into 6-day-old air-pouches was devoid of such an effect. This contrasts with rIL-1β administration, which produced neutrophil recruitment in both cavities. Pretreatment of the animals with dexamethasone abolished the chemoattractant effect of both cytokines.

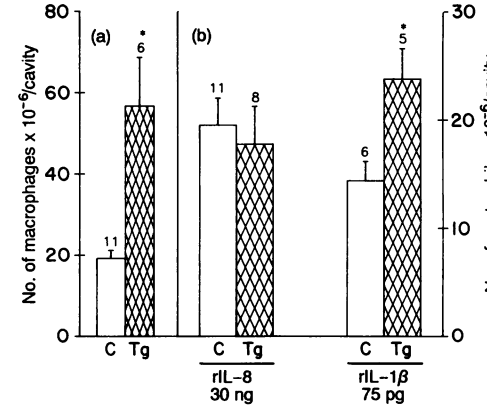

Figure 3. Thioglycollate pretreatment of rat peritoneal cavities potentiates neutrophil migration induced by rIL-1β but not by rIL-8. (a) Shows the macrophage population in PBS (C; open bar) and thioglycollate (Tg; crosshatched bar) pretreated cavities. (b) Shows the neutrophil migration induced by 30 ng of rIL-8 and 75 pg of rIL-1β in PBS (C) and thioglycollate (Tg) pretreated groups. The results are reported as mean ± SEM. The number of animals utilized is indicated at the top of each column. $*P<0.05$ compared to control (C); Student's t-test.

Figure 2 shows a significant decrease in the neutrophil migration induced by rIL-8 and rIL-1 when the resident peritoneal cell population was reduced by previous peritoneal lavage. This figure also shows that the neutrophil migration induced by FMLP was unaffected by the peritoneal lavage. On the other hand, when the peritoneal macrophage population was increased by pretreatment of the animals with thioglycollate, rIL-1β-induced neutrophil migration was significantly potentiated, while the effect of rIL-8 remained unaltered (Fig. 3).

Homologous transference of total peritoneal cells to the 6-day-old skin air-pouches rendered these cavities responsive to

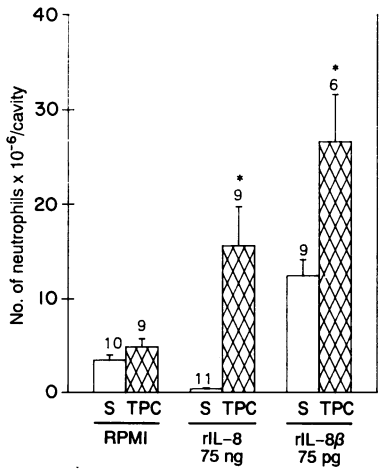

Figure 4. Previous transference of total homologous peritoneal cells renders rat air-pouches responsive to rIL-8 and potentiates neutrophil migration induced by rIL-1β. The bars represent neutrophil migration induced by RPMI, rIL-8 (75 ng) or rIL-1β (75 pg) into air-pouches of sham-washed animals (S; open bars) or into air-pouches previously injected with homologous total peritoneal cells (TPC; hatched bars). The results are reported as mean ± SEM. The number of animals utilized is indicated at the top of each column. $*P < 0.05$ compared to sham-operated animals (S); Student's *t*-test.

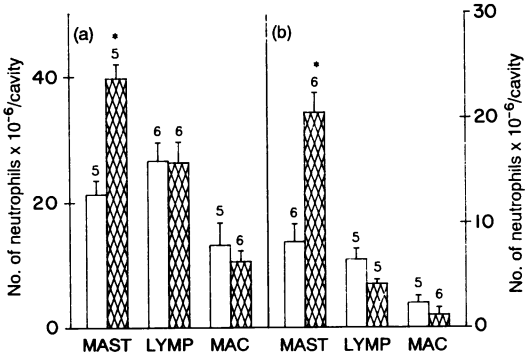

Figure 5. Release of neutrophil chemotactic factor from mast cells, lymphocytes and macrophages stimulated by rIL-8. The bars indicate the neutrophil migration into peritoneal (a) or air-pouch (b) cavities induced, respectively, by injection of 3 or 1 ml of the supernatant from mast cells (MAST), lymphocytes (LYMP) or macrophages (MAC) preincubated with medium (open bars) or rIL-8 (20 ng/ml, hatched bars). The results are reported as mean ± SEM. The number of animals utilized is indicated at the top of each column. $*P < 0.05$ compared to controls; Student's *t*-test.

the chemotactic effect of rIL-8 and potentiated the neutrophil emigration induced by rIL-1β (Fig. 4).

Figure 5 shows that the retentate of ultrafiltered supernatant of stimulated mast cells by rIL-8 induced neutrophil migration into peritoneal and air-pouch cavities. In contrast, the supernatants of rIL-8-stimulated lymphocytes and macrophages did not cause significant neutrophil migration in either cavities.

DISCUSSION

In the present paper we present evidence that rIL-8-induced neutrophil emigration is indirect and mediated by resident cells.

The contribution of resident cells to the effect of rIL-8 is supported by two observations:

(i) lavage of peritoneal cavities, which reduces resident cells but does not modify the responsiveness to direct chemoattractants such as FMLP, decreased neutrophil migration induced by rIL-8; and
(ii) the transference of peritoneal resident homologous cells to the air-pouch rendered this cavity responsive to rIL-8 stimulation.

Additional indirect evidence favouring this hypothesis is the observed reduction of rIL-8-induced neutrophil migration in dexamethasone-treated rats. In this context, it must be pointed out that in dexamethasone-treated animals[18] direct chemotactic stimuli continue to produce similar neutrophil migration to that observed in naive animals, while the effect of various substances like LPS, carrageenin, zymosan or IL-1, IFN-γ and TNF-α is abolished.[2,4,18] This dexamethasone inhibitory effect was explained as resulting from the blockade of the release of chemotactic factors, stimulated by inflammatory stimuli.[2,18] Thus, our results are in line with the suggestion that *in vivo* rIL-8 causes neutrophil migration by an indirect mechanism, possibly via the release of another cytokine by resident cells. This suggestion is further supported by the observation that IL-8 did not cause neutrophil migration into air-pouch cavities, a test which is sensitive to direct chemotactic substances (personal observation).[24] It must be pointed out, however, that our suggestion of an indirect effect of rIL-8 is in apparent contradiction with the described *in vitro* direct chemotactic activity of this cytokine.[5-7] Nevertheless it should be considered that, in order to promote *in vivo* neutrophil migration, in addition to its effect upon neutrophil locomotion, a direct active chemotactic substance must also be able to stimulate neutrophil/endothelium adhesion.[25] rIL-8, however, has an inhibitory effect upon neutrophil/endothelium adhesion. It has been described that rIL-8 inhibited *in vitro* neutrophil/endothelium adhesion,[10] and when injected intravenously in rabbits or rats reduced neutrophil migration to the inflammatory site.[26]

The present series of experiments suggest that IL-8 injected into the peritoneal cavities causes neutrophil migration by a dexamethasone-sensitive release of a chemotactic substance by resident cells. Alternatively, the fact that dexamethasone reduced the rIL-8-induced neutrophil migration could be interpreted as resulting from the corticoid effect upon neutrophil/endothelium adhesion. However, the dose of the corticoid used in these experiments did not inhibit neutrophil/endothelial cell adhesion induced by leukotriene B_4 and FMLP[25] and did not affect the neutrophil migration induced by direct acting chemoattractants,[18] but inhibited the release of chemotactic stimuli.[27,28]

The resident cells that mediate the effect of rIL-8 and rIL-1 seem to be different. This suggestion is supported by the fact that:

(i) peritoneal cavities as well as the 6-day-old air-pouches responded to rIL-1, but rIL-8 did not cause migration in the latter cavity; and
(ii) the increase of the macrophage population by previous thioglycollate intraperitoneal administration did not alter

rIL-8-induced neutrophil migration while it increased that provoked by rIL-1. It is important to realize that the cell population present in the unstimulated 6-day-old air-pouches is essentially macrophages.[19,29]

As the transference of resident peritoneal cells to the air-pouch rendered this cavity responsive to rIL-8, cells other than macrophages could be involved in rIL-8-induced neutrophil migration. We have tested the possibility that macrophages, lymphocytes or mast cells were able to mediate the *in vivo* effect of rIL-8 and found that purified peritoneal mast cells incubated with rIL-8 (but not lymphocytes nor macrophages) released into the supernatant a factor which stimulated neutrophil migration. It must be pointed out that this supernatant caused neutrophil migration in the air-pouches, a cavity which is unresponsive to rIL-8.

Finally, since both rIL-1 and rIL-8 were derived from *E. coli* there is the possibility that the indirect chemotactic effect of the recombinant cytokines used could be attributed to LPS contamination. However, we have previously demonstrated that the rIL-1-induced effects were not due to LPS contamination.[2] Furthermore, rIL-8 contamination with LPS can also be discarded since LPS produces neutrophil migration when injected into both peritoneal and air pouch cavities[4] and rIL-8 did not cause neutrophil migration into the pouches at a dose which was effective in the peritoneal cavities. Moreover, pretreatment of peritoneal cavities with thioglycollate potentiated the neutrophil migration induced by intraperitoneal administration of endotoxin[18] but did not affect that induced by rIL-8.

In conclusion our results show that both rIL-8 and rIL-1 are able to induce *in vivo* neutrophil migration by an indirect mechanism, probably dependent on the release of a chemotactic factor by resident cells. Mast cells seems to be at least in part responsible for rIL-8-induced neutrophil migration *in vivo*.

ACKNOWLEDGMENTS

We thank Rubens de Melo and Sergio R. Rosas for technical assistance. R. A. Ribeiro is grateful to Universidade Federal do Ceara for granting him a leave of absence to conclude his PhD research.

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